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The histology of gill and muscle of Indian Major Carp, *Catla catla* exposed with Triphenyl phosphate

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Abstract: **Triphenyl phosphate (TPP) is a phosphate ester flame retardant product, widely used as Plasticizer. TPP induced toxicity response in tissues of Indian major carp, *Catla catla* has been reported. Histological observations confronted detrimental morphological and anatomical alterations induced in vital organs like gill and muscle by sub - lethal toxicity of TPP. Histological studies revealed that the tissue impairment were found to be adverse with increase in concentration (0.25mg/lt , 0.5 mg/lt and 1 mg/lt) and exposure duration (0 - 15days) of TPP. As a conclusion, the findings of the present histological investigations demonstrates that the exposure of fresh water teleost fish, *Catla catla* to varying concentration of TPP caused moderate to adverse tissue impairment in gill and muscle. Hence, this plasticizer, TPP should be used in low ratio for its toxic effect to save our environment.**

Keywords: **TPP, *Catla catla*, Plasticizer, Toxic effect.**

1. INTRODUCTION

Plastics are often embedded with chemical additives, which alter the properties of material, protect them from weathering (or) fire (Stapleton *et. al.*, 2012). One of the most extensively used chemical additives as flame retardant and plasticizer are organophosphate compounds, which are distributed abundantly in various environmental compartments all over the world (Wei *et. al.*, 2015). One of the most consumed organophosphate compound is, TPP which is a phosphate ester, having much utility both as a flame retardant in polyurethane foam and as a plasticizer in hydraulic fluids, lacquers and varnishes (CPSC, 2005; Van der Veen and de Boer, 2012). Due to the fact that TPP is not bonded to manufactured products, it is relatively prone to be released into the environment through leaching, abrasion and volatilization (Wei *et. al.*, 2015). TPP has been found in a variety of environmental compartments Worldwide (Dodson *et. al.*, 2012; Marklund *et. al.*, 2005; Stapleton *et. al.*, 2009; Yadav *et. al.*, 2018; Zhong *et. al.*, 2018), including waste water treatment plants (Kim *et. al.*, 2017; Krzeminski *et. al.*, 2017) and surface waters (Guo *et. al.*, 2017a; Li *et. al.*, 2017; Shi *et. al.*, 2016). The presence of TPP in fishes collected from rivers were also well documented (Giulivo *et. al.*, 2017; Guo *et. al.*, 2017b). TPP may serve as an endocrine disruptor has been reported in recent studies (Kim *et. al.*, 2015; Liu *et. al.*, 2012; Liu *et. al.*, 2013; Liu *et. al.*, 2016; Zhang *et. al.*, 2014), which could cause developmental neurotoxicity (Sun *et. al.*, 2016) and cardiotoxicity (McGee *et. al.*, 2013) in fish. Hence, in the present investigation, an attempt has been made to observe histopathological changes in vital organs like gill, muscle, liver, stomach, intestine and kidney of Indian major carp *Catla catla*, exposed to sub – lethal concentrations (0.25mg/l, 0.5 mg/l, 1 mg/l) of TPP technical grade, for an exposure period of 15 days.

2. OBJECTIVE

To study the effect of toxicity response induced in the tissues pertaining to vital organs in *Catla catla* upon exposure to TPP, an organophosphate plasticizer. Pilot studies were done on the toxicological assessment of TPP upon exposure to aquatic organisms like fishes and microalgaes. According to literature, there were no elaborate works on the histopathological aspects of TPP on fishes. Therefore, the present investigation is carried out to study the effect of TPP upon exposure in *Catla catla*.

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The study emphasizes on,

1) Assessement of LC_{50}
2) Animal behaviour
3) Histopathological changes in gill and muscle.

3. MATERIALS AND METHODS

Animal selection and acclimatization:

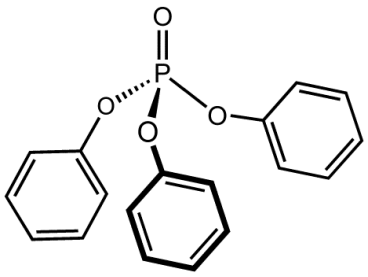
Catla catla, is the experimental animal model used in this study (fig 1) which belongs to the family of Cyprinidae of order cypriniformes. They are economically important South Asian fresh water fish, native to rivers and lakes. It is one of the most important aqua - cultured species. It is also grown in polyculture ponds with rohu and mrigal carp. It is a surface and mid water feeder. The experimental fish fingerlings of size (2 – 4 gm), were procured from Tamil Nadu Fish Seed Farm, Poondy, Thiruvallur and shifted in plastic bags containing fresh water filled with oxygen to the research laboratory. The fingerlings of fish were immersed in 0.1% $KMnO_4$, for 2 – 3 minutes in order to sterilize before acclimatization.

The process of acclimatization was carried out for a period of one week in glass aquaria of size 30cm x 60cm x 45 cm filled with water before the start of the experiment in the laboratory. Ten fish fingerlings were kept in each glass aquaria. The fish fingerlings were fed with commercial artificial feed at 2 - 3 % of wet body weight during the acclimatization period. Proper aeration was supplied continuously to all the glass aquaria with electric air pump. The water in the glass aquaria was replaced with fresh water for every two days.

Physio – Chemical Properties of TPP:

The present study involves TPP, technical grade as chemical, whose toxic effect in tissues of vital organs of Cattla carp has been evaluated.

Structure of TPP:



Appearance: Colourless crystalline Solid

Chemical Name: Triphenoxyphosphine oxide

CAS Number: 115-86-6.

Molecular Formula: $C_{18}H_{15}O_4P$

Molecular Weight: 326.29 g/mol

Water Solubility: 1.9 mg/l at room temperature.

Solubility in Other Solvents: TPP is soluble in other organic solvents such as benzene, chloroform, dimethyl ether, acetone and is moderately soluble in ethanol.

Melting point: 49℃

Boiling point: 370 - 500℃

Vapour pressure: 1.2×10-3 Pa at 20°C and 2.4×10-3 Pa at 25°C.

Fig 1: Photograph showing experimental model – fingerling of *Catla catla*

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Fig 2: Treated Fish

(showing swelling in abdomen and suffocation to breathe)

Determination of LC_{50}:

Determination of LC_{50} was done according to Behreus and Karbeur (1953). In the present study 72hrs LC_{50} bio – assay method was followed in which the ten fishes per group was placed at various concentrations of TPP in each group. The mortality rate was observed and recorded at time intervals of 24hrs, 48hrs, 72hrs. The concentration of TPP which gave 50% mortality at 72hrs was taken as the LC_{50} value. The percentage mortality was converted into probit values and plotted against the log dose values.

Experimental Design For Sub – Lethal Study:

The sub – lethal study was carried out by placing five groups which contain ten fishes per group and the group comprising of group – I – Control, group – II – Acetone treated, group – III – Sublethal treated with 0.25 mg/l of TPP, group – IV – sublethal treated with 0.5 mg/l of TPP and group – V – treated with 1 mg/l of TPP in 20 litres of water. The water in the glass aquaria was changed for every two days and freshly prepared toxicant was added to maintain the concentration of TPP at constant level. The experiment was carried out for a duration of 15 days.

General Observation:

The control and treated fishes were closely monitored for their,

1) General behaviour and body weight
2) Mortality
3) Signs of illness
4) Reaction to toxicant in tissue level

The observed changes were recorded under control group and experimental groups. The experiment was done for a period of 15 days. Photos were taken during the period of study. After the exposure of plasticizer TPP for 15 days, the animals were sacrificed and the tissues were subjected to histopathological studies.

Histopathological examination on gill and muscle tissues was carried out on the tissue samples of control and experimental groups of fishes exposed to sub lethal concentrations of TPP. Tissues were fixed in 10% neutral buffered formalin. Thin sections of 5µ thickness were prepared, stained with haematoxylin and eosin, observed under the light microscope.

Histopathological studies:

Histopathological studies were carried out on tissues taken from gill and muscle by dissecting the control and experimental fishes. Following procedure was adopted for histopathology: (Bancroft and stevens, 1977; Bancroft and Cook, 1984)

1. Fixation: Organ tissues were kept in Bouin solution for 24 hrs. Solution consisted of aqueous picric acid, formalin and glacial acetic acid in a ratio (75: 20: 5) respectively.

2. Dehydration: After fixation, tissues slices were kept in tissue baskets with tagging. Dehydration was done in Alcoholic ascending grade series as 30% (for 30 minutes), 50 %(for 30 minutes), 70%, 90% (for 2 hours each) and 100% for 2 hours two times.

3. Clearing: After dehydration, tissue baskets were transferred to clearing agent, Xylene for 1 hour and 2 hours.

4. Infiltration: After clearing, tissues were placed in molten paraffin wax for 45 minutes and 60 minutes at 58-69 °C in oven.

5. Embedding: Tissues were removed and embedded in moulds with paraffin wax. Wax blocks with embedding tissues were freezed for solidification.

6. Sectioning: Tissues were cut at 5 micron by using Microtome. Ribbons were spread on glass slide containing adhesive material as glycerine and albumin. Sections were placed in oven at 37°C.

7. Staining: Wax was removed from sections with xylene for 2 minutes for 3 times. Hydration was done by immersing the tissues in descending series of alcohol for 1-2 minutes each. (100%, 90%, 70%, 50% and 30%). Tissues were stained with haematoxylin stain for 2-5 minutes and then washed the slides under running tap water to remove excess stain. Counter stained with Eosin (1%) for 15 seconds to 2 minutes. Washed with water, dried in oven for 2 minutes. Mounted with Canada balsam.

8. Examination: Slides were examined under light microscope and photographed at 40 x10 X objective lens. The histopathological changes in experimental groups were observed and compared with that of control group.

4. RESULTS

Assessment of LC_{50}:

The LC_{50} of TPP in *Catla catla* was found to be 25 mg/l . The LC_{50} value was calculated by constructing the regression line, taking test doses and their corresponding mortalities in logarithmic values using Behreus and Karbeur (1953) probit analysis. The $1/25^{th}$ value of LC_{50} that is 1mg/l, $1/50^{th}$ value of LC_{50} that 0.5 mg/l and $1/100^{th}$ value of LC_{50} that 0.25 mg/l was chosen for sub lethal toxicity study.

General behaviour:

The control groups of the fishes were very normal whereas, *Catla catla* treated with sub lethal doses of TPP (0.25mg/l, 0.5 mg/l, 1 mg/l) showed signs of intoxication characterized by irregular, erratic and sometimes jerky movements. The fish exhibited a behaviour of trying to jump out from the treatment medium, to avoid the toxicant. The colour change in the skin from white to yellow was observed in the treatment group exposed for 15 days. Bulging of the abdomen, haemorrhage in the operculum region, reddening of eyes and suffocation to breathe was noticed, during the period of experimentation (Fig. 2).

Body weight:

The body weight of the fishes recorded during the study indicates that the control fishes show significant increase in body weight whereas the treated fishes showing decrease in the body weight. (Table. 1 and Graph 1).

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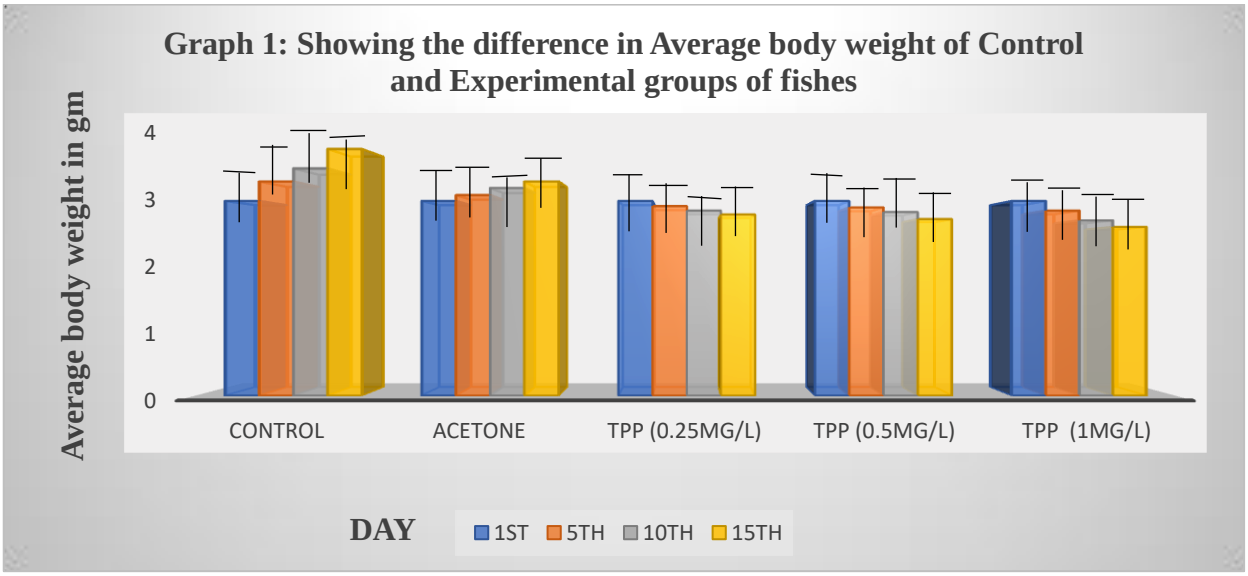
Table 1: Showing the body weight of both control fishes and experimental fishes

DAY	CONTROL FISH (Average weight in gm)	ACETONE TREATED FISH (Average weight in gm)	TPP(0.25mg/l) TREATED FISH (Average weight in gm)	TPP(0.5mg/l) TREATED FISH (Average weight in gm)	TPP(1mg/l) TREATED FISH (Average weight in gm)
1ST	3	3	3	3	3
5TH	3.20	3.09	2.92	2.90	2.85
10TH	3.50	3.20	2.85	2.83	2.70
15TH	3.80	3.30	2.79	2.72	2.60



HISTOPATHOLOGY:

Gill:

The control section of gill showed a normal histological appearance, (fig. 3). Whereas the gill section of *Catla catla* treated with sub lethal dosages of TPP (0.25mg/l, 0.5 mg/l, 1 mg/l) for 15 days showed rupture of gill lamellae, edema, hyperplasia, epithelial necrosis, disintegration of primary gill lamellae, enlargement of the secondary gill lamellae and fusion of secondary lamellae was seen in the gills (Fig .4, 5, 6 and 7).

Muscle:

In the control fish, normal architecture of the muscle was seen (Fig. 8). Whereas the muscle tissue of *Catla catla* exposed to sub lethal dosages of TPP (0.25mg/l, 0.5 mg/l, 1 mg/l) for 15 days , showed intercellular space, rupture of cell wall, fragmentation of muscle bundle, disruption of cells and the muscle bundles are separated. The vacuolation of cells are seen all over the focal area (Fig.9, 10, 11, and 12).

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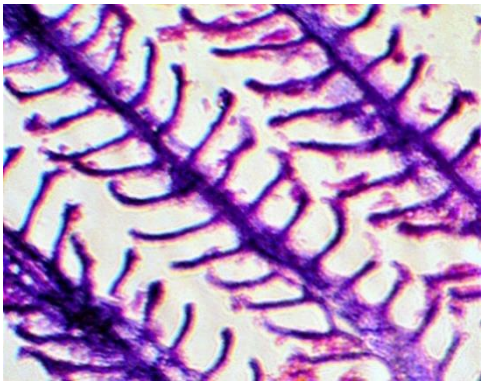
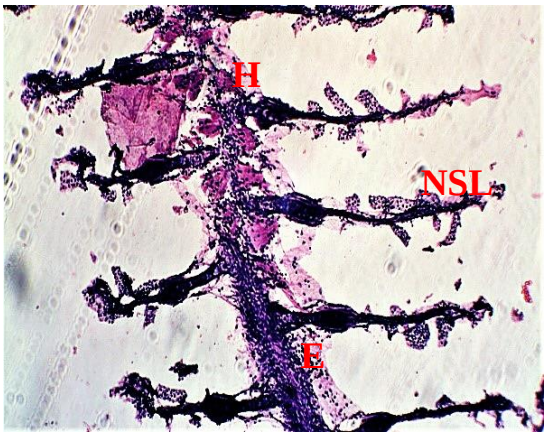
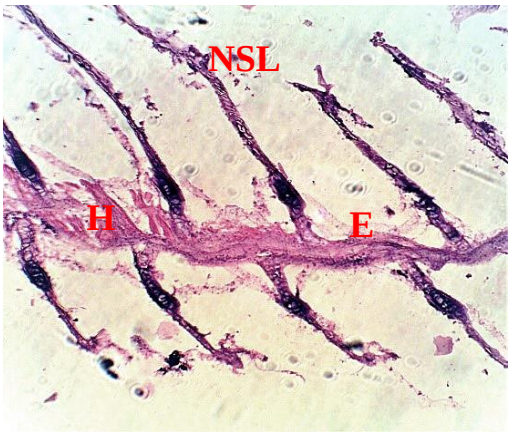


Haematoxylin and Eosin stained photomicrograph showing the histology of a Control gill tissue of *Catla catla* – (X 100) (Fig 3)

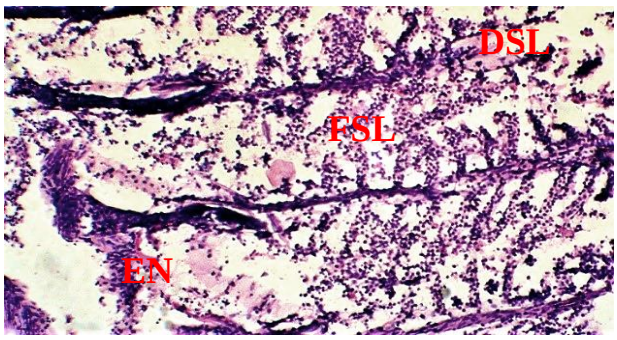
Haematoxylin and Eosin stained photomicrograph showing the histology of Acetone treated gill tissue of *Catla catla* - (X 100) (Fig 4). EN- Epithelial Necrosis, E – Edema, P – Primary Lamellae uprooted from their bases, H- Hyperplasia, D – Degeneration of primary Lamellae.



Haematoxylin and Eosin stained photomicrograph showing the histology of gill tissue of *Catla catla* treated with (0.25 mg/l) b.wt. of Triphenyl Phosphate - (X 100) (Fig 5). NSL – Necrosis of Secondary Lamellae E – Edema, H – Hyperplasia.



Haematoxylin and Eosin stained photomicrograph showing the histology of gill tissue of *Catla catla* treated with (0.5 mg/l) b.wt. of Triphenyl Phosphate - (X 100) (Fig 6). NSL – Necrosis of Secondary Lamellae, E – Edema, H – hyperplasia.



Haematoxylin and Eosin stained photomicrograph showing the histology of gill tissue of *Catla catla* treated with (1 mg/l) b.wt. of Triphenyl Phosphate - (X 100) (Fig 7).

FSL – Fusion of Secondary Lamellae, DSL – Disruption of Secondary Lamellae, EN – Epithelial Necrosis.

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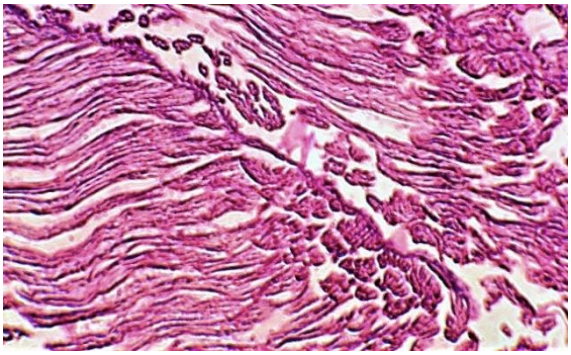
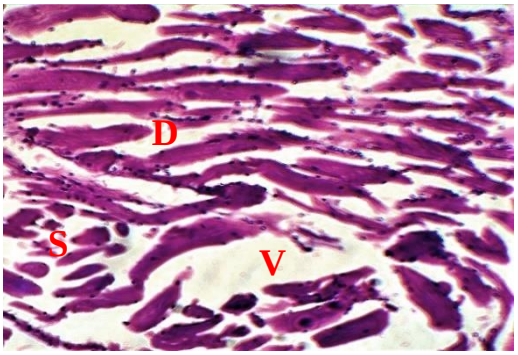
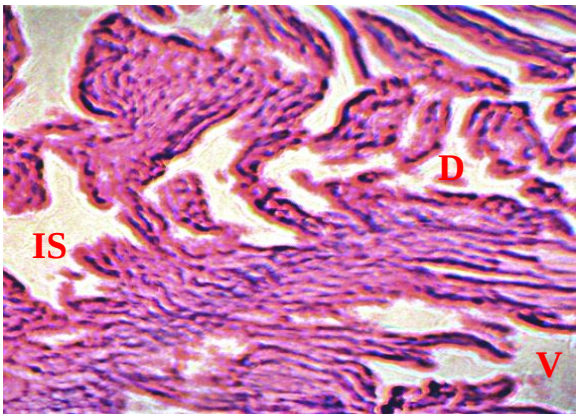


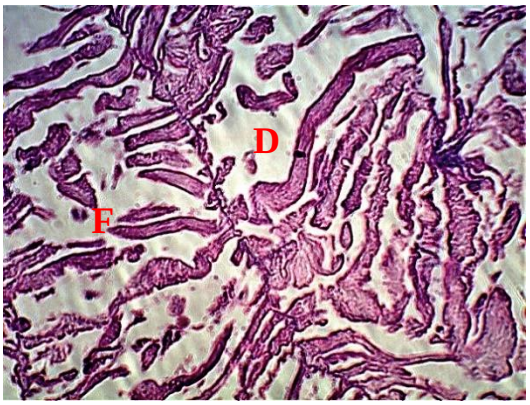
Haematoxylin and Eosin stained photomicrograph showing the histology of a Control muscle tissue of *Catla catla* – (X 100) (Fig 8)



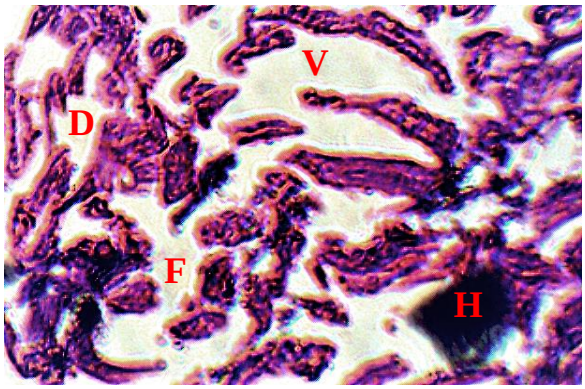
Haematoxylin and Eosin stained photomicrograph showing the histology of Acetone treated muscle tissue of *Catla catla* - (X 100) (Fig 9). D – Disruption of Muscle Bundle, S – Shortening of Muscle Bundle, V – Vacuolation.



Haematoxylin and Eosin stained photomicrograph showing the histology of muscle tissue of *Catla catla* treated with (0.25 mg/l) b.wt. of Triphenyl Phosphate treated - (X 100) (Fig 10). D – Disruption of Muscle Bundle, IS – Intercellular Space, V – Vacuolation.



Haematoxylin and Eosin stained photomicrograph showing the histology of muscle tissue of *Catla catla* treated with (0.5 mg/l) b.wt. of Triphenyl Phosphate -(X100) (Fig 11).
F – Fragments of Muscle Bundle, D – Disruption of Muscle Bundle.



Haematoxylin and Eosin stained photomicrograph showing the histology of muscle tissue of *Catla catla* treated with (1 mg/l) b.wt. of Triphenyl Phosphate - (X 100) (Fig 12).
H – Haemolysis, F – Fragments of Muscle Bundle, D – Disruption of Muscle bundle, V – Vacuolation.

5. DISCUSSION

Histological examination of the tissues is an important part of toxicity studies of chemical substances (Butler *et. al.*, 1981). In the present study the histopathology of gill, muscle, liver, intestine, stomach and kidney were studied. Plasticizers are mixed with polymers to increase flexibility of plastics. However, plasticizers are not covalently bound to plastics, and thus leach from products into the environment. Several studies have reported that plasticizers, induce adverse health effects in higher to lower vertebrates by direct (or) indirect contact. In the present investigation, TPP a plasticizer, found to cause extensive damage of the gill lamellae and complete dissociation of acidophilic cells was also observed. Lifting of the basement membrane was observed. Changes may be due to the result of the chemical reaching through the circulatory pathway and inflicting damage. Behavioural changes, such as erratic swimming may be due to the damage of gill lamellae. In the present study the gill section of *Catla catla* treated with sub lethal dosages (0.25mg/l, 0.5 mg/l, 1 mg/l) of TPP showed disruption of lamellae, hyperplasia, and epithelial necrosis, enlargement of secondary lamellae and fusion of secondary lamellae. Edema in the gill epithelium is also seen. (Ghate and Leelamulherkar, 1979) and (Gupta and Rajbanshi, 1995) also reported that the toxic substances causes damage to gill tissues, thereby reducing the oxygen consumption and disrupting the osmoregulatory function of aquatic organisms. The gills represent the print of closet proximity of the internal and external environments in terms of the diffusion distance from the water to the blood and large surface area exposed to the water. Consequently, histopathological changes in the respiratory epithelium of gill would ultimately affect the rate of oxygen uptake. Thus, direct contact of the respiratory surface area with polluted water may lead to its alteration as well as its diffusing capacity. Muscle tissue of *Catla catla* exposed to sub lethal doses (0.25mg/l, 0.5 mg/l, 1 mg/l) of TPP showed fragments of muscle bundles, inter cellular space, vacuolation, rupture of cell wall and disruption of muscle cells. The doses of TPP used in the present study are insignificant when compared to their environment persistent levels. Thus, the findings from the present study envisage that TPP has a potential to induce hazardous response in aquatic organisms when they come in contact with them either through direct or indirect mode. It also signifies that TPP are prone to be toxic to human kind also when they are bio – accumulated in the long run.

6. CONCLUSION

This study is a small piece of work which strongly says that TPP is a harmful plasticizer, when used for long years. Its residue make hazardous effects to the vital organs of fish, amphibians, mammals etc,. Knowingly or unknowingly a minimal amount of this TPP which is a hazardous plasticizer enters into the food chain and humans are the ones who consume these fishes every often. So, the end user like humans are more prone to this particular chemical. Hence use of this plasticizer can be controlled to a maximum level to safe guard our eco system and provide this land a eco-friendly land to the next generation.

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